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Kirchman, David L. and Dexter, Stephen C.

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Proteins are important in regulating microbial fouling of surfaces placed in seawater. Our ONR accomplishments are: 1) using an immuno-assay, we showed that the algal protein, ribulose-1,5-bisphosphate carboxylase (RuBPCase) is dissolved in seawater at concentrations higher than expected for a single protein; 2) a new method for measuring combined amino acids (includes proteins) in seawater was shown to measure higher concentration than the old method; 3) the basic mechanism and factors governing RuBPCase adsorption in seawater were described; and 4) the relationships among surface wettability, degradation of adsorbed protein, and bacterial growth were examined. In the future, we propose: 1) To determine, using immuno-assays, how (if at all) an immobile cell can degrade adsorbed protein not in contact with the cell; and 2) To examine, using transposon mutagenesis, the possible relationship between attachment and nutritional requirements of the attaching bacterium.

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June 28, 1989

Yearly Progress Report

Contract N00014-87-K-0108 R&T Code 441h001

Principal Investigators: David Kirchman and Stephen Dexter

Contractor: University of Delaware

Contract Title: Protein Adsorption and its Role in Bacterial Film Development

RESEARCH OBJECTIVES: To investigate the mechanisms of protein adsorption, the effect of adsorption on protein conformation, and relationships among surface properties, protein conformation, and bacterial film development on surfaces placed in seawater.

PROGRESS: We have been working in the following areas: 1) mechanisms of protein adsorption in seawater; 2) the use of immunological and HPLC techniques to examine dissolved and adsorbed proteins in seawater and their degradation by bacteria in seawater; 3) the relationship between adsorbed proteins and growth of attached bacteria; 4) the use of a lectin to examine natural surfaces possibly colonized by bacteria.

We have completed our work on the mechanisms of protein adsorption in seawater. This work has been described by Kirchman et al. (in press) and by last year's progress report. Below we summarize briefly our progress in the other areas.

I. Characterizing Dissolved and Adsorbed Proteins in Seawater by Immunological and HPLC Techniques

An Immunological Study of Dissolved Proteins in Seawater Study of organic films and their effect on microbial film development is hampered by our ignorance of marine organic chemistry. With present methodology, we can identify roughly 20% of the dissolved organic matter (DOM) present in seawater. New methodology has revealed that previous estimates of total dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) may be low by a factor of 2 to 5 (Suzuki et al. 1985; Sugimura and Suzuki 1988), so the fraction of DOM that can be assigned a chemical name may be even lower than 20%. Dissolved combined amino acids, which includes proteins (see below), can be a large fraction of total DON and their supply regulates bacterial growth both free living and attached to surfaces (Kirchman et al. in prep.; see below). During the first phase of our ONR project, we worked on several methodology problems with studying proteins in seawater.

Because the polypeptides dissolved in seawater probably reflects that of the plankton (specifically phytoplankton), the number of polypeptides dissolved in seawater is potentially very large. Thus, in order to conduct controlled

experiments, we needed to focus on a single protein, rather than work with the complex and probably uncontrollable mixture found in seawater. We focussed on the CO_2-fixing enzyme, ribulose-1,5-bisphosphate carboxylase (RuBPCase), because it is present in all autotrophs (Miziorko and Lorimer 1983) and is said to be the most abundant enzyme in nature. Because of the large number of possible proteins dissolved in seawater, immunological assays appeared to be the only feasible approach to measure the concentration of RuBPCase and to follow its fate in seawater. Polyclonal antibodies against the large subunit of RuBPCase were developed and tested for their specificity. The antisera used during this study was found to be specific against the large subunit of RuBPCase in chlorophyll c-containing algae, which include the most abundant phytoplankton genera in the oceans.

In order to use the immuno-assay, it was necessary to concentrate the dissolved organic matter. We explored several methods. The best method consisted of using tangential flow ultrafiltration and a membrane with a molecular weight cutoff of 10,000 daltons. This cutoff is adequate because even the small RuBPCase subunit is about 15,000 daltons. The recovery efficiency of added protein was about 75% when 0.1% SDS was used. Total protein was also measured by the BCA assay. RuBPCase was detected by a simple dot-blot assay based on alkaline phosphatase. We also have used a ^{3}H-labelled secondary antibody, which increases sensitivity.

We found that RuBPCase was detectable in seawater samples from the Delaware Bay. RuBPCase roughly covaried with total protein, but RuBPCase concentrations were on the order of ng/liter whereas total protein concentrations were on the order of µg/liter. Protein concentrations were about 10% of dissolved combined amino acids (DCAA; see below). Although occurring in low concentrations, RuBPCase is more abundant than if all possible proteins occurred at equal concentrations, i.e. pg/liter.

Several controls were performed to ensure that RuBPCase (or some antigenic determinants preserved on fragments of RuBPCase) was actually measured. First, Western blot analyses have shown that the antisera is specific for the large subunit of RuBPCase and that cross-reactions with other polypeptides are negligible. Second, pre-immune sera from the rabbit gave negative results. Third, no cross-reactions were detected if only the secondary antibody conjugated to alkaline phosphatase was used.

Combined Amino Acids as Measured by HPLC We are interested in a simple, direct assay for proteins in seawater that does not depend on concentrating the sample. Chemical oceanographers measure, by high performance liquid chromatography (HPLC), the amino acids released from acid hydrolysis (dissolved combined amino acids; DCAA) which includes polypeptides, but also amino acids complexed with other material, such as clay. We explored a new method for hydrolyzing DCAA, after talking with Herb Waite (U.Delaware), a protein biochemist. The "new" method was developed by Tsugita et al. (1987) for laboratory samples of proteins, but we are the first to apply it to seawater. We found major differences between the old and new methods of hydrolysis. Our work showed that the most important difference between the two methods is that, in the new metnod, the sample is first dried down and then hydrolyzed by acidic vapors,

whereas in the old method, the sample is hydrolyzed directly in 6N HCl. In both methods, the amino acids released by acid hydrolysis are analyzed by HPLC.

We found that the new method consistently measured higher DCAA concentrations compared with the old method. In the Delaware Bay, the difference is over 2-fold. Both methods give the same answer when proteins are added to seawater controls. There is no obvious blank problem with the new method--in fact, blanks are often lower. The amino acid composition, as determined by HPLC, does not appear to differ substantially between the two methods, although the new method recovers some amino acids more effectively than the old method. Preliminary measurements suggest that the difference is not as great as for samples from offshore. We hypothesize that the proteins from terrestrial sources are complexed with structural material from higher plants, which reduces the efficiency of the standard acid hydrolysis. These protein-containing complexes would be more abundant in estuaries than in offshore waters.

We used our new method for measuring combined amino acids to examine the adsorption of naturally-occurring combined amino acids to surfaces with different wettability (related to hydrophobicity). We found that the amount of adsorbed combined amino acids was higher on the low energy surfaces, as predicted by the laboratory experiments with RuBPCase. Furthermore, the percent of hydrophobic amino acids was higher on the low energy surfaces compared with the high energy surfaces.

II. Effect of Adsorbed Proteins on Bacterial Colonization and Growth

We measured colonization rates of natural bacterial assemblages to bare surfaces and to protein-coated surfaces. During experiments over the spring, summer and fall, we have found every possible result; adsorbed proteins can enhance, inhibit or have no effect on bacterial colonization. The cause of this variability is not clear, and there are no obvious explanations, such as season, temperature, or general biological activity. Given the variability observed among single bacterial species, it appears that changes in bacterial assemblages result in the different effects of adsorbed proteins on bacterial colonization.

Another reason why proteins may have a complex effect on bacterial colonization is the patchy distribution of adsorbed proteins on surfaces. Adsorbed proteins can be visualized directly with the fluorochrome FITC. One problem, however, is that FITC can bind nonspecifically to bare surfaces. We have been using a more specific method, indirect immuno-fluorescence. Surfaces were coated with the RuBPCase and probed with antibodies that bind specifically to RuBPCase. Cross-reactions are detected by using secondary antibodies that are conjugated to FITC, which allows visualization of the adsorbed protein with epifluorescence microscopy. The protein patches fluoresce green with blue light excitation. Attached bacteria can be simultaneously stained with the fluorochrome DAPI, which fluoresces blue with excitation at 365 nm. Using this double-stain technique, it is clear that proteins do not adsorb evenly over surfaces and that bacteria colonize bare spots as frequently as protein patches. In fact, if we assume a reasonable size and concentration for proteins

occurring naturally, the amount of adsorbed protein is not large enough to form a protein monolayer over the surface.

Although the effect of adsorbed proteins on bacterial attachment (initial minutes to hours) is variable and complex, the effect on bacterial growth is clear: over longer time periods (>6 h), bacterial biomass is higher on protein-coated surfaces. This result has been observed consistently in many experiments. We hypothesize that while bacterial attachment (the initial events) may be inhibited, the growth of those bacteria attaching to the protein-coated surface is enhanced. To test this hypothesis, the biomass production of day-old bacterial films was estimated from the incorporation of ^{3}H-thymidine. These experiments clearly show that bacterial production is higher on protein-coated surfaces. We also observed that bacterial production was higher on low energy surfaces (low work of adhesion). One simple explanation is that protein adsorption is higher on low energy surfaces.

However, the absolute amount is not the only factor determining how proteins affect bacterial growth. Another factor is the availability of the adsorbed protein. We found that percent removal by proteases was higher for high energy surfaces (e.g., glass) than for low energy surfaces (e.g., plastics). Also, percent degradation of the adsorbed protein by the marine bacterium Pseudomonas S9 was higher on high than on low energy surfaces. We hypothesize that the protein-surface bond is stronger on low energy surfaces compared to high energy surfaces. This seems likely because our earlier studies showed tnat hydrophobic interactions were most important in determining protein adsorption (Kirchman et al. in press).

We next tested how the growth of Pseudomonas S9 supported by adsorbed proteins varied with surface energy. For cells that have been attached to surfaces for <4 h, the growth rate (as measured by thymidine incorporation) was unmeasurable on low energy surfaces and increased with increasing surface energy. Other experiments indicate that growth rates on low energy surfaces increase as the protein is degraded. These results are consistent with previous experiments on percent degradation of adsorbed proteins. Bacterial growth rates are initially low on low energy surfaces because the protein-surface bond decreases the availability of the adsorbed protein. Eventually, the adsorbed protein is degraded to support increased bacterial growth.

III. Lectins and Natural Surfaces: A New Method for Measuring Chitin

Chitin is abundant in nature and may be an important component of naturally-occurring surfaces such as suspended detrital particles and aggregates. However, study of its role in natural environments has been hampered by the lack of an adequate assay for measuring concentrations. Our work on this problem is clearly "boot-legged" and not part of our main objectives. Nevertheless, it represents an interesting and perhaps important transition from our previous work (Kirchman et al. 1982) to our proposed work on lectins. We hypothesize that proteins such as lectins are key to specific mechanisms by which bacteria recognize and adhere to favorable substrata. Although there has been much work on specific attachment mechanisms in

biomedical areas, the existence of analogous mechanisms in marine bacteria and natural ecosystems has not been explored.

Previous methods for measuring chitin relied on chemical or enzymatic hydrolysis of a sample with subsequent quantification of N-acetylglucosamine (NAG), the monomeric unit of chitin. Chemical hydrolysis is not adequate because cell walls of bacteria, which would be present in all environmental samples, contain large amounts of NAG. Chitin assays relying on chitinase may underestimate chitin concentrations because chitinase may be ineffective in complex environmental samples with low chitin concentrations.

We developed a simple, inexpensive, and specific assay for chitin that relies on the lectin, wheat germ agglutinin (WGA). Lectins are a general class of proteins that bind to carbohydrates. The lectin WGA has been used extensively in histological work to localize chitin (Tronchin et al. 1981; Peters and Latka 1986; Greven and Peters 1986). There is no published work that examines the use of WGA to estimate chitin quantitatively. Briefly, our method is as follows: the lectin is ^{3}H-labelled by borohydride and is added to samples. After incubation, the samples are filtered and the filters radioassayed. The amount of radioactivity associated with the filter is directly proportional to the amount of chitin in the sample. Current detection limit is 200 ng per sample. For a typical measurement, this means that the particles in about 100 ml are concentrated over 0.2 μm Nuclepore filters before addition of ^{3}H-WGA. Detection limits could be improved several fold by increasing the specific activity of the WGA (e.g., simply use more ^{3}H-borohydride). ^{125}I-labelling would greatly increase sensitivity, but the assay would be more difficult to use because of the hazards of gamma radiation from ^{125}I.

We performed many controls to examine the specificity of our assay for chitin in the presence of other macromolecules and detritus. Most importantly, WGA does not bind to cellulose even at high concentrations. Cellulose is structurally quite similar to chitin. We used the WGA assay to examine chitin concentrations in the Delaware Bay and in the subarctic Pacific.

Future Work Plan: We plan several experiments to clean up and finish the work discussed briefly above. Also, we are particularly interested in further experiments with Pseudomonas S9 to examine how (if at all) a firmly adhered, non-motile cell can degrade adsorbed protein not directly adjacent to the cell. The strategy of excreting exoproteases may be advantageous in the two dimensional world of attached bacteria. We plan on trying to track excreted exoproteases and adsorbed proteins with immuno-gold.

We also would like to examine whether or not marine bacteria have specific attachment mechanisms. We will examine whether or not Pseudomonas S9 has a specific attachment mechanism to protein patches adsorbed to surfaces. One approach we plan on using is to obtain transposon mutants that are deficient in either exoprotease or attachment or both. Similar experiments are planned to examine attachment of Vibrio sp. to chitin.

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Students Supported by ONR Project

Dan McDonald, M.S. Candidate

Lucy Feingold, M.S. Candidate

Award

Kirchman was elected Chairman of Division N (Aquatic and Terrestrial Microbiology), American Society of Microbiology, effective July 1, 1989.

BELAS, M. Robert
Center of Marine Biotechnology
University of Marylandsity
600 East Lombard Street
Baltimore, MD 21202

BLAKE, III, Robert C.
Department of Biochemistry
Meharry Medical College
Nashville, TN 37208

BLAKEMORE, R. P.
Department of Microbiology
University of New Hampshire
Durham, New Hampshire 03824

BURCHARD, Robert P.
Department of Biological Sciences
Univ of Maryland-Baltimore County
Catonsville, MD 21228

CHAPMAN, David J.
Department of Biology
UCLA
405 Hilgard Avenue
Los Angeles, CA 90024

CLARK, Douglas S.
Dept of Chemical Engineering
University of California
Berkeley, CA 94720

COLWELL, Rita
Maryland Biotechnology Institute
University of Maryland
Microbiology Building
College Park, MD 20742

COOKSEY, Keith E.
Department of Microbiology
Montana State University
Bozeman, MT 59717

DANIELS, Charles J.
Department of Microbiology
Ohio State University
484 West 12th Avenue
Columbus, OH 43210

DANIELS, Lacy
Department of Microbiology
University of Iowa
College of Medicine
Iowa City, IA 52242

DENNIS, Patrick P.
Department of Biochemistry
University of British Columbia
2146 Health Sciences Mall
Vancouver, B.C. V6T 1W5

DOOLITTLE, W. Ford
Department of Biochemistry
Dalhousie University
Halifax, Nova Scotia
CANADA B3H 4H7

EISENBERG, Henryk
The Weizmann Institute of Science
Dept of Polymer Research
P.O. Box 26
Rehovot 76100, Israel

EPEL, Dvid
Hopkins Marine Station
Stanford University
Pacific Grove, CA 93950

FELBECK, Horst
Marine Biology Research Division
Scripps Institution of Oceanography
University of California – San Diego
La Jolla, CA 92093

FISHER, Charles R.
Marine Science Institute
University of California-Santa
Barbara
Santa Barbara, CA 93106

GIBOR, Aharon
Marine Science Institute
University of California
Santa Barbara, CA 93106

GONZALEZ, Elma
Department of Biology
UCLA
Los Angeles, CA 90024

GREENBERG, Everett P.
Department of Microbiology
University of Iowa
Iowa City, Iowa 52242

GUNSALUS, Robert P.
Department of Microbiology
UCLA
405 Hilgard Avenue
Los Angeles, CA 90024

GUPTA, Ramesh
Southern Illinois University
Dept of Chem and Biochemistry
Carbondale, IL 62901

HASTINGS, J. Woodland
Biological Laboratories
Harvard University
16 Divinity Avenue
Cambridge, MA 02138

HAYGOOD, Margo
Marine Biology Research Division
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92093

JENSEN, Roy A.
Department of Microbiology
University of Florida
Gainesville, FL 32611

KELLY, Robert M.
Dept of Chemical Engineering
The Johns Hopkins University
Baltimore, MD 21218

KIRCHMAN, David L.
College of Marine Studies
University of Delaware
Robinson Hall
Newark, DE 19716

KONISKY, Jordan
Department of Microbiology
University of Illinois
809 Sout Wright Street
Champaign, IL 61820

LEADBETTER, Edward R.
Dept of Molecular and Cell Biology
University of Connecticut
Box U-131
Storrs, CT 06268

LIAO, Hans H.
Biotechnology Center
University of Wisconisn
1710 University Avenue
Madison, WI 53705

LIDSTROM, Mary E.
Keck Laboratories 138-78
California Institute of Technology
Pasadena, CA 91125

MITCHELL, Ralph
Division of Applied Sciences
Harvard University
125 Pierce Hall
Cambridge, MA 02138

MORSE, Daniel E.
Marine Science Institute
University of California
Santa Barbara, CA 93106

NADATHUR, Govind S.
Marine Science Institute
Univ Cal-Santa Barbara
Santabarbara, CA 93106

NEALSON, Kenneth H.
Center for Great Lakes Studies
University of Wisconsin-Milwaukee
600 E. Greenfield Avenue
Milwaukee, WI 53204

OLSEN, Gary J.
Indiana University
Department of Biology
Jordan Hall 138
Bloomington, Indiana 47405

PACE, Norman R.
Department of Biology
Indiana University
Bloomington, IN 47405

PREZELIN, Barbara B.
Marine Science Institute
University of California
Santa Barbara, CA 93106

REEVE, John N.
Department of Microbiology
Ohio State University
484 West 12th Avenue
Columbus, OH 43210-1292

ROSEMAN, Saul
Department of Biology
Johns Hopkins University
Baltimore, MD 21218

SEARCY, Dennis G.
Zoology Department
Univeristy of Massachusetts
Amherst, MA 01003

SILVERMAN, Michael
Agouron Institute
505 Coast Blvd. South
La Jolla, CA 92037

SMIT, John
Department of Microbiology
University of British Columbia
#300 - 6174 University Blvd
Vancouver, British Columbia
V6T 1W5 CANADA

SPUDICH, John L.
Dept of Anat and Structural Biolgy
Albert Einstein College of Medicine
1300 Morris Park Avenue
Bronx, NY 10461

STAHL, David A.
College of Veterinary Medicine
University of Illinois
Urbana, IL 61801

SWIFT, Hewson
Dept of Molec Genetics
and Cell Biology
University of Chicago
1103 East 57th Street
Chicago, IL 60637

TAYLOR, Gordon T.
Hawaii Institute of Geophysics
University of Hawaii
2525 Correa Road
Honolulu, HI 96822

TOSTESON, Thomas R.
Department of Marine Sciences
University of Puerto Rico
Mayaguez, PR 00709

TRENCH, Robert K.
Marine Science Institue
University of California-Santa
Barbara
Santa Barbara, CA 93106

WALEH, Nahid
Molecular Biology Department
SRI International
333 Ravenswood Avenue
Menlo Park, CA 94025

WHITE, David
Institute of Applied Microbiology
University of Tennessee
P. O. Box X, Building 1503/6
Oak Ridge, TN 37831

WOESE, Carl R.
Genetics Department
University of Illinois
515 Morrill Hall
Urbana, IL 61801

YAYANOS, A. Aristides
Physiological Research Laboratory
Scripps Institution of Oceanography
University of California-San Diego
La Jolla, CA 92093

ZINDER, Stephen H.
Department of Microbiology
Cornell University
Stocking Hall
Ithaca, NY 14853